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Evaluation of antifungal activity of vitavax and *Trichoderma viride* against two wheat root rot pathogens

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Abstract
Objective: This study evaluated the effectiveness of applying the chemical "vitavax", the biological control product "plant guard" and the fungal isolate *T. viride* to control two wheat root rot pathogens, *Drechslera biseptata* and *Fusarium moniliforme in vitro* and *in vivo*.
Methodology and results: Vitavax, plant guard and non-sterilized culture filtrate (NSCF) of *Trichoderma viride* completely inhibited the growth and sporulation of *F. moniliforme* when applied at high doses. Hyphal extension and sporulation of *D. biseptata* ceased in medium amended with higher doses of NSCF of *T. viride* while amending the culture medium with 4% plant guard completely inhibited sporulation only. Infestation of soil with either pathogen was associated with root rot of wheat seedlings, decreasing emergence of seedlings and low values of growth parameters of wheat root and shoot. Photosynthetic pigments, carbohydrates, proteins and phenolic compounds in the host tissues decreased due to infection. Application of chemical and biological control, either alone or in combination, improved the biochemical parameters in the treated seedlings. Mechanisms of action may include induction of host resistance as was observed from the improved emergence and growth of seedlings as well as reduced disease severity. *T. viride* was most effective in controlling the two pathogens, both *in vitro* and *in vivo*.
Conclusion and application of findings: Chemical and biological control were efficient against root rot pathogens *in vitro* and *in vivo*. Application of *T. viride* alone or in combination with vitavax is a promising approach for managing wheat root rot.

Keywords: Wheat, *Drechslera biseptata*, *Fusarium moniliforme*, vitavax, plant guard, *Trichoderma viride*, growth activities, biochemical changes

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Introduction
Drechslera biseptata and *Fusarium moniliforme* are the causal agents of root rot diseases of wheat and several other crop plants. Fungicides have been used widely to control these pathogens *in vitro* (Reuveni, 2006) and *in vivo* (Errampalli, 2004). The fungicide bavastin inhibited conidial germination and sporulation of *Fusarium oxysporum* (El-Abyad *et al*., 1983), as well as growth (Sarhan *et al*., 1999). Vitavax 200B increased germination of wheat seeds and reduced seedling infection by *Cochliobolus sativus* (Sharma-Poudyal *et al*., 2005).

Besides being expensive and unsuitable for the environment, conventional chemical methods of disease management

are not totally effective and may lead to the appearance of new resistant strains of phytopathogens (Bruin & Edgington, 1981). It is now widely recognized that biological control offers suitable alternatives for plant disease management (Wiest *et al*., 2002).

Many isolates of *Trichoderma* sp. control plant pathogens through secretion of antifungal compounds (El-Mehalawy, 1999) or parasitism (Bae & Knudsen, 2007). Plant-Guard, a commercially available product constituting spores of *Trichoderma harzianum* when applied together with rizo-N (*Bacillus subtilis*) significantly inhibited growth of phytopathogens (El-Habbaa *et al*., 2002). Dinakaran *et al*. (1995) reported reduced intensity of root rot disease on sesame seed treated with *T. viride* and *T. harzianum.* Some isolates of *T. virens* have been effective in suppressing damping-off disease caused by *Rhizoctonia solani* under greenhouse conditions (Roberts *et al*., 2005).

The integration of biocontrol agents and fungicides has been effective against root rot and collar rot diseases (Tewari & Mukhopadhyay, 2003). Integrated treatments also protected several crops against seed and soil-borne pathogens better than the individual application of either biocide or fungicide (Dubey, 2003).

This study was conducted to evaluate the effectiveness of applying the chemical "vitavax", the biological control product "plant guard" and the fungal isolate *T. viride* to control two wheat root rot pathogens, *Drechslera biseptata* and *Fusarium moniliforme in vitro* and *in vivo*.

Materials and methods

Pathogens and host plants: The two root rot fungi used in this study, *Drechslera biseptata* (Sacc. & Roum) Richardson & Fraser and *Fusarium moniliforme* Sheld were isolated from diseased wheat roots and maintained on potato dextrose agar (PDA) medium. Seeds of wheat (*Triticum aestivum* L.) cultivar Yecora rejo were obtained from the Regional Agriculture and Water Research Centre, Riyadh, Saudi Arabia.

Pest control agents: The fungicide vitavax contains the active ingredients carboxin (5, 6-dihydro-2-methyl-N-phenyl-1,4-oxathiin-3-carboxamide) (17% w/v) and thiram (tetramethylthiuram disulfide) (17% w/v). The recommended dose is 2 ml kg^{-1} seeds. Stock solutions of vitavax were prepared and diluted to obtain concentrations of 4, 8, 16 and 32 µg active ingredient per milliliter of medium.

The bioagents included the biocide 'plant guard' and the antagonist *Trichoderma viride*. Plant guard is a commercial formulated solution containing spores of *Trichoderma harzianum* ($30x10^6$ spores ml^{-1}). The recommended dose is 10 ml L^{-1} for seed treatment.

T. viride was obtained from the MERCEN Centre of Microbes Collection, Faculty of Agriculture, Ain-Shams University, Egypt. The fungus was first grown in potato dextrose broth (PDB) at 27°C for 7 days. Part of the culture filtrate was sterilized by passing through 0.45µm bacterial filter (SCF) and the other part left without sterilization (NSCF).

Known volumes of plant guard or the culture filtrate of *T. viride* were added to the medium to give concentrations of 0.5, 1.0, 2.0 and 4.0% (v/v). The concentration of 1.0% contained $30x10^6$ spores/100 ml medium.

Pathogen growth and sporulation: Molten cooled PDA medium was mixed aseptically with the control agent to produce the required concentration and poured into Petri dishes. The PDA plates were inoculated with mycelial discs (10 mm diameter) of the pathogen and incubated at 27°C. The colony diameter was measured and the number of spores produced was counted using a haemacytometer after 9 days of growth (El-Abyad *et al*., 1983).

Management of root rot disease: Plastic pots (12 cm diameter) were filled each with an appropriate amount of autoclaved soil. The soil consisted of clay: sand: *betimos* (1: 1: 1 v/v). After growing on sterilized barley grains at 27ºC for 3 weeks *D. biseptata* or *F. moniliforme* were mixed with the soil at 4% (w/w). The soil was adjusted to 60% of its moisture holding capacity using sterile distilled water. Seeds were surface disinfected in 0.01% $HgCl_2$ for 30-60 s, then rinsed thoroughly with sterile distilled water. Before sowing the seeds were treated with vitavax (2 ml kg^{-1} seeds), or soaked in 1% plant guard or 1% NSCF of *T. viride* for one hour. For combined treatments,

seeds were soaked in 1% plant guard or NSCF of *T. viride* and thereafter, treated with vitavax. Seeds without any treatment served as control. Ten wheat seeds were sown in each pot, 5 days after infesting the soil with fungal inoculum. All pots were kept in greenhouse at 27±3°C. Data on seedling emergence and infection, as well as growth parameters of root and shoot (weight and length) were recorded. In addition biochemical analysis of photosynthetic pigments, total carbohydrates, proteins and phenolics were carried out 6 weeks after sowing.

Biochemical analysis: Photosynthetic pigments, including chlorophyll a, chlorophyll b and carotenoids contents of leaf, were estimated according to Lichtenthaler (1987). To measure total carbohydrates and phenolics the root and shoot tissues were oven dried at 80°C and extracted in soxhelt unit using 75% ethyl alcohol for 10-12 hrs. The extract was filtered and evaporated at 60°C. The dried residue was dissolved in a known volume of 50% isopropyl alcohol to determine the total carbohydrates by anthrone method as described by Umbriet *et al*. (1959), and total phenols according to Malik and Singh (1980).

The method reported by Bollag and Edelstein (1993) was used to estimate total protein content. Dried sample was ground in liquid nitrogen with 1 ml of phosphate buffer at pH 7.2 and traces of antioxidant ascorbic acid being added during grinding. The mixture was centrifuged at 18000 rpm for 20 min. A known volume of the supernatant was added to 3 ml serva blue G stain and the absorbance measured spectrophotometrically at 595 nm.

Statistics: All the results are average of at least three replicates. The data were analyzed by One-Way ANOVA ($p \leq 0.05$) (SPSS, 1999).

Results

In vitro efficacy of pathogen control agents: The chemical and biological control agents significantly reduced hyphal extension and sporulation of both *D. biseptata* and *F. moniliforme* (Fig.1). The control agents inhibited sporulation of fungi more than mycelial growth, with the number of spores produced and colony diameter being inversely proportional to the concentrations of pesticide applied. Vitavax, plant guard and non-sterilized culture filtrate of *T. viride* (NSCF) prevented growth and sporulation of *F. moniliforme* at the higher doses. However, growth of *D. biseptata* ceased only in medium amended with NSCF of *T. viride* at 2 and 4%. *D. biseptata* failed to produce spores in media amended with plant guard or NSCF of *T. viride* at the higher doses. Among the control agents, *T. viride* (NSCF) showed the most promising activity against both pathogens.

Emergence and infection of seedlings: Emergence of seedlings differed depending on the pathogen inoculated. *F. moniliforme* significantly reduced emergence, but *D. biseptata* appeared to have no significant effect compared to the untreated healthy control (Table 1). Treatment of wheat seeds with the control agents did not significantly alter emergence of seedlings in absence of pathogens. However, application of control agents in presence of pathogen significantly increased seedling emergence when compared with the control plants treated with pathogen only. Wheat cv. Yecora rejo was highly susceptible to infection by both pathogens, with 100% incidence. Brown and dark discoloration appeared at the base of the shoot as well as at the upper part of the root, later, these parts became rotten (Fig. 2). Chemical and biological control as well as combination of both reduced the number of infected seedlings (Table 1) and disease severity (Fig. 2). Application of *T. viride* in combination with vitavax provided the best protection to wheat seedlings against the selected root rot pathogens, with the infection incidence reduced to 10%. The application of plant guard alone was better than when combined with vitavax, with disease incidence of 19 and 12% in case of plant guard alone, compared to 40 and 34% in case of plant guard combined with vitavax in soil infested with *D. biseptata* or *F. moniliforme*, respectively.

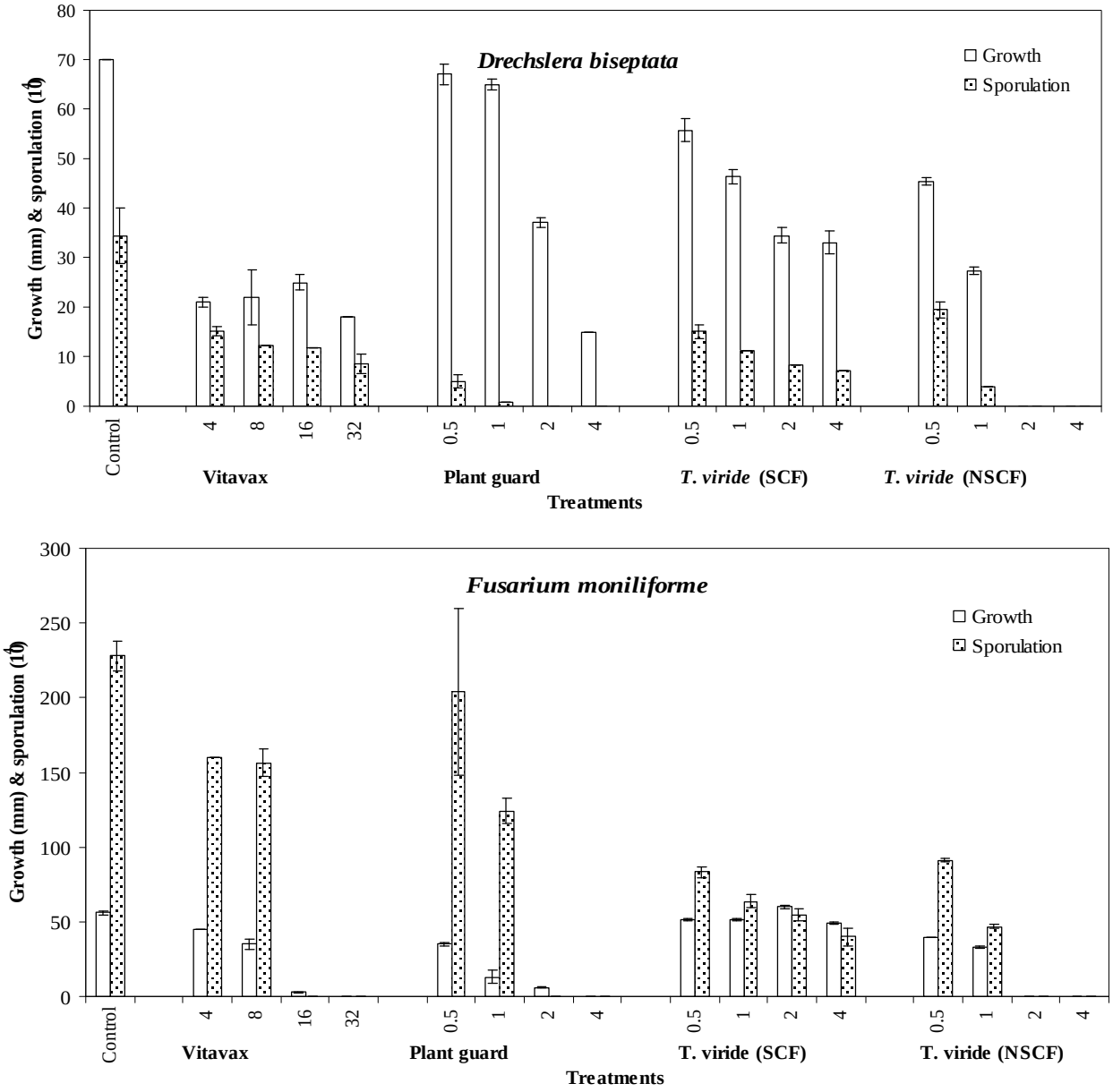

Figure 1: Growth (mm) and sporulation ($\times 10^4$) (over 9 days at 27°C) of *Drechslera biseptata* and *Fusarium moniliforme* on PDA medium supplemented with different concentrations of control agents.

Growth of wheat root and shoot: Infestation of soil with pathogens significantly decreased growth parameters (weight and length) of root and shoot. Treatment of wheat seeds with the control agents improved growth parameters of healthy and infected seedlings, when compared with the corresponding healthy and infected control, respectively (Table 2).

D. biseptata reduced seedling growth more than *F. moniliforme*, whereas the later pathogen exhibited greater activity against seedling emergence. In addition, pathogen effect was stronger on the root system than on the shoots .Generally, the control agents were effective in improving growth of roots of plants that were infected by *F. moniliforme* more than roots of plants that were infected by *D. biseptata*. However, the control agents were more effective in stimulating growth of shoots of plants that were infected by *D. biseptata* than on plants infected by *F. moniliforme*, as compared to the corresponding infected control. *T. viride* was the most efficient agent in improving the growth parameters of wheat seedlings

growing in soil infested with either one of the tested pathogens.

Photosynthetic pigments: The two root rot pathogens decreased photosynthetic pigments (chlorophyll a and b and carotenoids), whereas, the control agents increased them in leaves of healthy and infected seedlings when compared with the corresponding untreated control (Table 3). Plant guard, vitavax and *T. viride* were the most efficient agents in increasing photosynthetic pigments in healthy leaves, and leaves infected by *D. biseptata* and *F. moniliforme*, respectively.

Carbohydrates, proteins and phenolics content: The pathogens significantly decreased the carbohydrates, proteins and phenolic compounds contents in root and shoot tissues (Table 4). Data showed that control agents, particularly biocides, were capable of increasing these compounds in healthy and infected seedlings as compared to the control treatments. The highest carbohydrates and phenolic compounds contents were in tissues of seedlings treated with combined plant guard and vitavax, and *T. viride* alone, respectively. Plant guard was the most efficient agent in stimulating protein synthesis in healthy and in *F. moniliforme*- infected roots, whereas the application of vitavax resulted in the highest value in *D. biseptata*- infected root. However, the combination of *T. viride* with vitavax led to the accumulation of the highest amount of protein in healthy and infected shoots.

Table 1: Emergence and diseases incidence on wheat seedlings following application of control agents in absence and presence of either *Drechslera biseptata* or *Fusarium moniliforme* after 45 days from sowing.

Treatment	Seedling emergence (%)			Infection percentage (%)		
	Without pathogen	*D. biseptata*	*F. moniliforme*	Without pathogen	*D. biseptata*	*F. moniliforme*
Control	96	90	80	0	100	100
Vitavax	100	100	94	0	32	26
Plant guard	98	98	94	0	19	12
Plant guard & vitavax	96	98	98	0	40	34
T. viride	96	94	98	0	20	20
T. viride & vitavax	100	92	94	0	10	10
LSD 1%		10.6			15	
5%		7.5			10.5	

Discussion

Fungicide (vitavax), biocides (plant guard and *T. viride*) applied singulalrly or in combination suppressed the two wheat root rot pathogens *D. biseptata* and *F. moniliforme*. The degree of suppression varied with the pathogen and its developmental stage, as well as the type of control agent and its concentration. Similar trends have been reported by Kucuk and Kivanc (2003) and El-Mehalawy (2003).

The inhibitory effect of vitavax on growth and sporulation of the tested pathogens *in vitro* was directly proportional to its concentration, which agrees with the findings of El-Habbaa *et al*. (2002). Fungicides control pathogenic fungi by altering and inhibiting cell metabolism. Carboxin has been reported to reduce glucose oxidation to 50%, acetate oxidation to 70-90% as well as reducing the synthesis of DNA, RNA and protein by upto 60-90%. These effects inhibit fungal growth to about 90% (Ragsdale & Sisler, 1970). Some fungicides inhibit electron transfer between cytochrome b and cytochrome c in the respiratory chain (von Jagow & Link, 1986). This severely reduces the production of aerobic energy, thereby inhibiting fungal growth (Leinhos *et al*., 1997).

The biocides (plant guard and *T. viride*) were also effective against the two pathogens under investigation *in vitro*. El-Habbaa *et al*. (2002) and Dubey *et al*. (2007) have previously reported the antifungal activity of plant guard, *T. harzianum* and *T. viride*. In our study, competition between the bioagent *T. viride* (NSCF) and the pathogens for nutrients and space was observed on solid medium. At the higher concentrations *T. viride* colonized more space and prevented growth of the two pathogens. Such competition might be accompanied by other mechanisms of action, e.g. mycoparasitism and/or production of antifungal compounds as reported by Melo (1991) and Kucuk and Kivanc (2004).

Non sterilized culture filtrate of *T. viride* had higher antifungal activity than the sterile culture filtrate. This may be attributed to the presence of spores together with fungal metabolites in NSCF, whereas SCF contained fungal metabolites only.

Infestation of soil with the pathogens retarded emergence and growth of wheat seedlings. This outcome is expected as similar observations have been by Caruso *et al*. (1999). The virulence of the pathogen might be attributed to the production of gibberellins which could facilitate penetration of the host by stimulating cell elongation (Phinney & West, 1960).Production of cell wall degrading enzymes (El-Abyad *et al*., 1996) and mycotoxins (McLean, 1996) may also have an important role in pathogenicity, partly by helping the pathogens to overcome host defense mechanisms (El-Mehalawy, 2003). In this study the suppression of plant growth was accompanied by decreasing amounts of photosynthetic pigments, carbohydrates, proteins and phenolic compounds. A similar decrease in chemical contents in host tissues due to infection has been previously reported by Biswas *et al*. (2003) and Khilare *et al.* (2004). Gogoi *et al*. (2001) found that the amounts of proteins and phenolic compounds accumulated in susceptible plants were less than in resistant plants. Reduced production of host enzymes due to infection would facilitate fungal colonization of the host tissues (Miller & Greenhalgh, 1988). McLean (1996) concluded that mycotoxins could reduce growth of the host and leaf chlorophyll content and might also inhibit DNA, RNA and protein synthesis.

Seed treatment with the control agents decreased damage caused by the pathogens, as indicated by reduction of disease incidence and severity. Moreover, seedling emergence and growth were enhanced. Vitavax (Dubey, 2003), *Trichoderma* spp. as well as integration of *Trichoderma* and fungicide (Dubey *et al*., 2007) have controlled diseases in several crops. The ability of *Trichoderma* to stimulate growth of root and shoot may be attributed to inhibition of pathogen growth, detoxification of its toxin, production of plant growth hormones and vitamins, conversion of nutrients in a form useful to the plant, releasing nutrients from soil or organic matters and increasing uptake as well as translocation of minerals (Sarhan *et al*., 1999).

In this study there was a marked increase in photosynthetic pigments, carbohydrates, phenolic compounds and proteins contents of healthy and infected wheat plant when the seeds were treated with fungicide, biocide or a combination of both. This increase in biochemical contents of wheat root and shoot was accompanied by increasing seedling emergence and growth as well as reducing disease severity. Certain biochemical alterations in the host might be associated with induction of host resistance and enhancement of growth (Biswas *et al*., 2003).

The results conclusively demonstrate that the two root rot pathogens *D. biseptata* and *F. moniliforme* can be effectively managed by chemical and biological control *in vitro* and *in vivo*. The bioagent *T. viride* (NSCF) was the most effective among other control agents against both pathogens. Plant guard was not as effective as *T. viride* (NSCF) in controlling pathogens, possibly due to the longer time taken by conidia to germinate (Hjeljord *et al*., 2002). In addition, formulated conidia are more likely to come under nutrient stress, causing fungistasis, a condition that is unlikely to affect fresh conidia which have the ability to colonize host tissues more easily. Moreover, plant guard contains conidia only but *T. viride* (NSCF) contains conidia and antifungal compounds. These findings prove that for some biological control agents there is a relationship between antagonism *in vitro* and biological control *in vivo* as postulated by Aggarwall *et al*. (2004).

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Table 2: Growth parameters of 45-day-old wheat root and shoot following application of agents to control root pathogens *Drechslera biseptata* and *Fusarium moniliforme.*

Treatment	Fresh weight (mg/seedling)						Dry weight (mg/seedling)						Length (cm)					
	Without pathogen		*D. biseptata*		*F. moniliforme*		Without pathogen		*D. biseptata*		*F. moniliforme*		Without pathogen		*D. biseptata*		*F. moniliforme*	
	Roots	shoot	Roots	shoot	Roots	shoot	Roots	shoot	Roots	shoot	Roots	shoot	Roots	shoot	Roots	shoot	Roots	shoot
Control	99.2	572.8	46.0	293.4	70.0	407.0	16.7	80.0	7.6	42.0	10.4	48.9	13.0	28.4	6.8	26.3	7.0	26.8
Vitavax (A)	145.9	636.7	78.7	561.7	129.0	669.3	22.0	80.8	10.7	80.5	17.0	83.4	14.0	29.7	10.5	28.9	13.6	31.0
Plant guard (B)	122.2	590.1	67.9	538.1	122.3	591.5	16.9	87.6	8.5	76.8	16.3	78.2	14.5	29.8	9.7	29.5	13.7	29.5
A + B	106.2	581.7	97.1	668.0	114.3	578.6	11.4	67.9	10.7	84.9	12.4	69.6	14.4	29.0	9.7	30.1	13.2	28.8
T. viride (C)	103.6	652.7	99.1	730.7	136.6	694.4	22.3	97.5	11.0	105.9	17.0	85.5	14.8	29.3	11.8	31.1	14.1	31.2
A + C	119.0	793.1	98.5	573.5	120.6	632.3	12.8	101.5	9.8	77.6	14.7	80.9	14.0	30.3	9.5	28.5	13.2	29.0
LSD 1%	18.6	193.3					2.0	6.7					8.4	1.9				
5%	13.1	139.5					1.4	4.8					6.0	1.4				

Table 3: Effect of chemical and biological control of *Drechslera biseptata* and *Fusarium moniliforme* on photosynthetic pigments contents (mg/ g fresh wt.) in 45-day-old wheat leaves.

Treatment	Chlorophyll a			Chlorophyll b			Carotenoids			Total pigments		
	Without pathogen	*D. biseptata*	*F. moniliforme*	Without pathogen	*D. biseptata*	*F. moniliforme*	Without pathogen	*D. biseptata*	*F. moniliforme*	Without pathogen	*D. biseptata*	*F. moniliforme*
Control	4.3	1.3	0.8	1.6	0.9	0.8	1.1	0.9	0.6	7.0	3.1	2.2
Vitavax (A)	4.9	3.5	2.4	1.6	1.6	1.7	1.3	1.1	0.2	7.8	6.2	4.3
Plant guard (B)	5.5	2.4	4.2	1.7	1.1	1.6	1.5	0.8	1.1	8.7	4.3	6.9
A + B	4.4	2.4	2.9	1.4	1.2	1.3	1.2	0.7	0.9	7.0	4.3	5.1
T. viride (C)	4.8	2.7	4.6	1.6	1.2	2.7	1.3	1.0	1.2	7.7	4.9	8.5
A + C	5.4	3.2	3.6	1.4	1.4	1.6	1.4	1.1	0.9	8.2	5.7	6.1
LSD 1%		0.23			0.17			0.74				
5%		0.16			0.12			0.52				

Table 4: Effect of chemical and biological control of *Drechslera biseptata* and *Fusarium moniliforme* on total carbohydrates, proteins and phenols contents in 45-day-old wheat plants.

Treatment	Total carbohydrates						Total proteins						Total phenols					
	Without pathogen		*D. biseptata*		*F. moniliforme*		Without pathogen		*D. biseptata*		*F. moniliforme*		Without pathogen		*D. biseptata*		*F. moniliforme*	
	Roots	shoot	Root	shoot	Roots	shoot	Roots	shoot	Root	shoot	Roots	shoot	Roots	shoot	Root	Sht	Root	shoot
Control	0.91	4.0	0.27	2.73	0.35	3.6	0.92	4.97	0.29	3.09	0.61	2.55	0.33	3.1	0.19	2.0	0.32	1.9
Vitavax (A)	1.15	6.89	0.24	2.3	0.33	3.4	1.28	5.31	1.09	4.47	1.39	3.07	0.86	3.1	0.34	2.4	0.49	2.0
Plant guard (B)	0.95	6.6	0.36	2.43	0.39	3.47	1.53	4.99	0.52	3.6	1.84	2.91	0.74	3.3	0.41	2.0	0.45	2.1
A + B	1.47	7.0	0.75	4.27	0.9	5.37	1.14	5.05	1.03	4.0	1.19	3.97	0.49	3.5	0.4	2.3	0.47	2.3
T. viride (C)	0.92	4.2	0.55	2.87	0.67	5.1	1.0	5.26	0.81	3.81	1.0	3.73	1.39	3.3	0.5	2.4	0.62	2.8
A + C	1.27	4.48	0.35	3.07	0.36	4.47	0.98	5.59	0.78	4.48	0.94	4.79	0.45	3.1	0.35	2.3	0.4	2.4
LSD 1%	0.025	0.7					0.3	0.39					0.123	0.04				
5%	0.018	0.49					0.2	0.27					0.087	0.03				

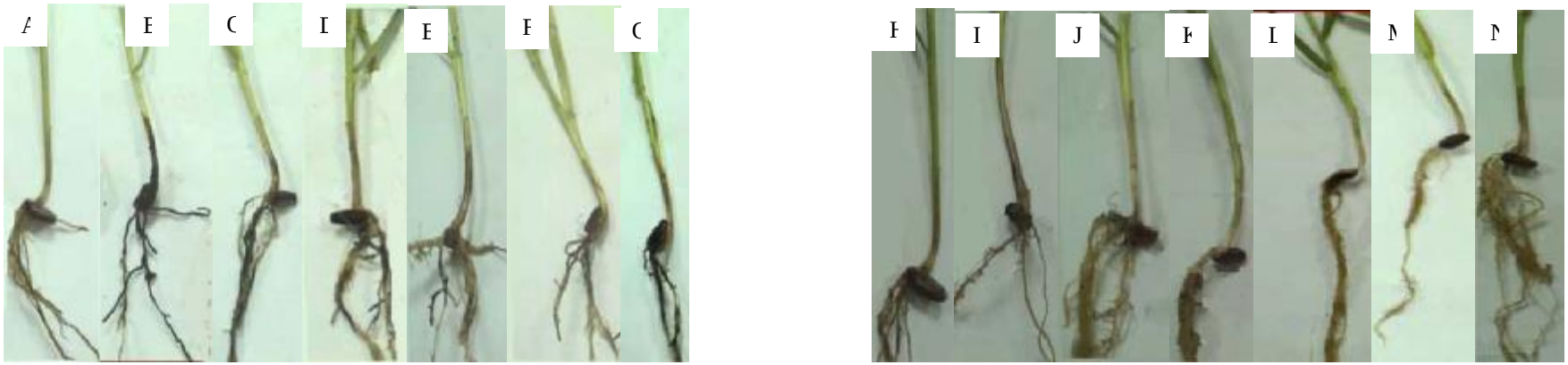

Figure 2: Reduction of wheat root rot disease symptoms caused by *Drechslera biseptata* (A-G) and *Fusarium moniliforme* (H-N) following application of control agents. Images are A=control; B=*D.biseptata* (Db) alone; C=Db + vitavax; D=Db + plant guard; E=Db+plant guard+vitavax; F=Db+T.viride; G=Db+T.viride+vitavax; H=control; I=*F.monoliforme* (Fm) alone; J=Fm+ vitavax; K=Fm+ plant guard; L=Fm+plant guard+vitavax; M=Fm+T.viride; N=Fm+T.viride+vitavax.

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